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Pheochromocytoma (PC 12) as a Model Cell Line for Membrane Permeabilization Studies in the presence of Electromagnetic Fields (EMFs): Recent Advances

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Abstract

The pheochromocytoma PC 12 cell line is an established model system for neurosecretion and neuronal differentiation, particular to study cellular responses to nerve growth factors (NGF) and how these lead to expression of differentiation-specific proteins and differentiation. More recently, PC 12 has become a model system for investigating cell membrane permeabilization and cell attachment on different substrata. Of particular interest is the use of PC 12 to study the fundamental responses of cells to electromagnetic fields (EMFs) of 18 GHz and THz in the range of 0.3-19.5×10^{12} Hz, a type of radiation treatment shown to induce membrane depolarization and transient increase in permeability with no changes in cell viability, morphology, proliferation and cellular physiology. This makes EMFs of 18 GHz and THz radiation a promising alternative to conventional poration techniques for drug and gene delivery applications. This article will review recent progress in the use of PC 12 to investigate EMF radiation-induced cell membrane permeability, as well as to study mammalian cell attachment preferences and differentiation on polymer surfaces, including those coated with high molecular weight proteins of the extracellular matrix, e.g. laminins, poly-l-lysine, fibronectin, and on novel metallic surfaces of nanostructured titanium.

Pheochromocytoma (PC 12) Cells

Neuronal cell models have been extensively used for mechanistic studies and for the detection of potential neurotoxicants[1]. Clonal cell lines that can express neuronal properties can be used as a model to study the potential response of the nervous system to different types of stimuli[2,3]. Pheochromocytoma (PC 12) is one such cell line that was originally derived in 1976 from a pheochromocytoma rat tumour, a type of tumour originating in the chromaffin cells of the adrenal medulla, by Greene and Tischler[2,4].Since their discovery, PC 12 cells have evolved into a valuable model for studying cell signalling, particularly because this cell line is able to respond to several growth factors, hormones and neurotrophins[4].More recently, PC 12 cells have been used as a model cell line to study the response of mammalian cells to EMFs of 18 GHz, a type of EMF radiation known to induce membrane permeabilization with minimal detriment to cell viability, morphology, proliferation and cellular physiology. For this application, PC 12 cells offer ease of culture and handling, and more importantly, PC 12 cells exhibit distinct responses with regard to differentiation, cellular proliferation and survival, which can be assessed independently[4]. PC 12 cell line has been used widely to

study the neuroprotective effects of drugs and components of traditional Chinese medicines on cells previously injured by a neurotoxin, such as 1-methyl-4-phenylpyridinium ion (MPP^+), or due to oxidative damage from compounds such as H_2O_2[5]. This is because PC 12 cells have neuron-like properties due to their ability to respond to nerve growth factor (NGF) by differentiating into sympathetic ganglion neurons[1]. Furthermore, being a cancer-like cell line, PC 12 can be sub-cultured indefinitely, providing experiments with desired reproducibility[3]. Upon treatment with NGF, PC 12 cells cease proliferation, and initiate the process of differentiation by extending neurites as well as become electrically excitable[1,6]. Most studies that explore the mechanism of PC 12 cell differentiation in response to NGF tend to monitor its effect in an exponentially growing cell population[7]as the population responds to NGF in an identical manner[7].

PC12 as a model for EMF-induced cell membrane permeabilization

There is a growing body of evidence suggesting that treatments using certain types of EMFs have the ability to heal surface wounds and skin lesions, promote bone regeneration after fractures and reduce pain and swelling after surgical interventions[8]. However, decoupling the underlying molecular mechanisms by which exposure to EMF induces these favourable medical outcomes is far from trivial. Of all possible biological targets of EMFs, the cell plasma membrane has been identified as one of the most important targets for such treatment. In particular, the exposure of cells to EMF treatment has been shown to alter the rate with which ion channels open and close, as well as have a direct effect on the magnitude of ion fluxes via these channels[9]. Since the plasma membrane provides a selective barrier that controls what enters and exits the cell, the potential consequences of EMF treatment-induced changes in membrane permeability can be profound[10-12]. In contrast, there is a great deal of debate as to whether EMF radiation can penetrate the cell membrane, since many believe that the nature of EMFs would make it difficult for this type of radiation to cross the cell plasma membrane[13].

Considering the large amount of background knowledge with respect to PC 12 proliferation and differentiation in response to different stimuli, PC 12 was chosen as a preferred cell line to study the fundamental biological effects of EMF exposure on cells, both with respect to transient changes in cell membrane permeability, as well as the ability of EMF to directly induce intracellular damage.

Classification of electromagnetic field radiation

The European Commission grouped the sources of non-ionising radiation depending on their source and frequency[14], namely:

(i) Radio frequency fields (RF),

(ii) Intermediate frequency fields (IF),

(iii) Extremely low frequency fields (ELF),

(iv) Static fields.

EMF induced effects on PC 12 cells at high frequencies (RF)

Exposure of PC 12 cells to EMFs of 18 GHz for 30 s with a specific absorption rate (SAR) of 1.17 $kWkg^{-1}$ led to a transient increase in the cell membrane permeability for up to 9 min in up to 90 % of the treated cells[15]. In order to prevent bulk heating, cells were exposed in three cycles, with 2 min cooling periods in between. The reversible membrane permeabilization was demonstrated by the rapid internalization of silica nanospheres (d = 23.5 nm) and their clusters (d = 63 nm) by EMF-exposed cells within the first 9 min after treatment. In contrast, PC 12 cells exposed to bulk heating and the untreated control did not exhibit nanosphere internalization. Following the EMF exposure, the growth and cell morphology of PC 12 cells were studied. The PC 12 cells were able to remain viable after the exposure. In order to gain a deeper insight into the changes in cell metabolism as a result of EMF treatment, biochemical assays were performed on the EMF exposed cells. It was found that the level of metabolic activity of PC 12 cells subjected to the above EMF treatment regimen was similar to that of the heat-treated cells and control cells that received no treatment. Specifically, the total protein concentrations and the level of lactated dehydrogenase (LDH) release were not significantly different between the three experimental groups. The study concluded that the 18 GHz EMF treatment may induce transient permeabilization of cell membrane in a mammalian cell model without any detrimental effect to cell viability or metabolism, thereby providing a potential alternative to conventional poration techniques in drug delivery application. In another study conducted, PC 12 cells were permeable in the presence of THz radiation, increased permeability was evidenced by an increased uptake of silica nanospheres (d = 23.5 nm) and their clusters (d = 63 nm) in comparison to the untreated control sample[16]. To date, most nanotechnology based cancer therapies have focused on the treatment of primary tumours but it is of utmost importance to leverage the potential of nanotechnology to fight cancer spread at all stages of the metastatic process. The methods that are currently effective work on treating large, well-vascularized tumours; yet these methods are not as efficient when dealing with small clusters of malignant cells[17,18]. The results of the study showed that subsequent THz exposure was able to internalise both individual nanospheres and their clusters. Enhanced uptake of silica nanosphere clusters was observed. This is important because nanoparticles used in nanotechnology based cancer treatments in the range of 1 – 1000 nm[17]. Therefore, the

EMF based method has promising prospects in terms of clinical application of nanospheres as therapeutic carriers delivering drugs to single or a small cluster of cells at all stages of the metastatic process. For example, silica nanoparticles have recently entered clinical trials for the detection of lymph node metastases in patients suffering from melanoma using PET (positron emission tomography)[19]. EMF would provide an effective alternative to techniques currently used to promote gene and drug transfer that includes electroporation, sonoporation, microinjection, impalefection, biolistic gene transfer, magnetofection, hydroporation, and photoporation[20,23].

The behaviour of PC 12 cells in response to this type of EMF radiation was consistent with that of other eukaryotic and prokaryotic cells types treated with 18 GHz EMFs[15], suggesting that the mechanism of EMF-induced increase in permeability is not likely to originate from bulk heating of the suspension since exposure of cells to similar temperatures in the absence of microwave radiation failed to induce cell permeabilization. This leads us to believe that the observed transient increase in PC 12 cell membrane permeability is likely to be electro-kinetic in nature. It is likely to arise as a result of the increased conductivity and mobility of ions across cell membrane, with potential contribution from microthermal changes that cannot be easily captured at the macro level, as well as from the direct interaction of the EMF with cell membranes and/or their structural and functional components (*e.g.*, phospholipids)[24-26]. Several possible reactions that can take place inside a cell upon exposure to EMF have been discussed[8]. It has been suggested that EMFs change the membrane structure and thus its penetrability to small molecules and ions, *e.g.*, Ca^{2+}. The local changes in ion concentrations may be caused by local changes in pH and temperature, which can also induce reorganization of cyto skeletal elements. Interestingly, in PC 12 cells treated with 18 GHz EMFs, only a transient increase in trans-membrane transport was observed, with no evidence of changes in the cytoskeleton or motor proteins, or proliferation rate compared to control cells. For example, the degree of polymerization of microtubules, the tubular polymers of a dimer of two globular proteins, alpha and beta tubulin, maybe disturbed by intermediate-frequency (100–300 kHz) electromagnetic radiation[8]. Together with microfilaments and intermediate filaments, microtubules provide structural support for the cell. Microtubules also serve as an enabling platform for intracellular transport and play an important role in movement of secretory vesicles, organelles, and intracellular macromolecular assemblies, as well as in cell division. Thus, by interfering with microtubule polymerization, EMF may affect cytoskeleton physiology, leading to changes in intracellular transport as well as a decreased rate of proliferation due to EMF-induced disruption in chromosome alignment and separation, mitosis aberrations, and eventual mitotic arrest in cells.

EMF induced effects on PC 12 cells at extremely low frequency (ELF) fields

Power transmission lines or electrically powered devices transmit ELF fields, consisting of frequencies of 50 or 60 Hz that are quasi-stationary, and allow the electric and magnetic fields to be analysed separately, together with their effects on organisms[8,14,27]. In a study conducted by[28], significant (20% enhancement) neurite outgrowth at a frequency of 50 and 70 Hz was reported in comparison to the control sample at other frequencies (1, 10, 30 and 100 Hz). It was also reported in another study that ELF-EMFs (50 Hz) affect proliferation and neurogenesis of PC 12 cells[29]. That data showed that differentiating and non-differentiating PC 12 cells appeared more vulnerable to ELF-EMFs while fully differentiated PC 12 cells were more stable[29].

PC 12 as a model to study cell attachment and differentiation

The attachment of PC 12 cells on various substrata and their response to Nerve Growth Factors (NGF), including the release of differentiation-specific proteins, have been widely studied.

PC 12 cell attachment on substratum

Studies have found that there is a wide variety of factors that can influence PC 12 axonal outgrowth under *in vivo* conditions. These include the nature of chemotactic and trophic factors, *e.g.*, NGF, concentration gradients[30,31], contact guidance[32], and the physicochemical interaction of actively differentiating nerve fibres with the different types of substrata encountered during the elongation process[30-33]. Using PC 12 cells, the physical and chemical properties of the substratum were shown to play a critical role in determining the growth pattern of neurons[32]. Cellular growth and differentiation requires the presence of a structured environment with which cells can interact[34]. It has been previously suggested that the neuronal growth cone tends to select the path that offers least resistance, and to migrate in spaces or 'groves'[35]. This theory was supported by several studies where PC 12 cells was shown to adhere differently on to various surfaces. For example, when PC 12 cells were allowed to attach and differentiate on surfaces coated with proteins typically found in the extracellular matrix, i.e. laminin (Lam), poly-l-lysine (PLL), fibronectin (Fn) or a combination of thereof, over a period of five days, increased attachment of PC 12 cells was observed on substrata containing Lam and a combination of PLL/Fn proteins[30]. The extent of neurite outgrowth was strongly dependent on the nature of the substrate, with substrata possessing dual coatings (PLL/Lam and PLL/Fn) exhibiting the highest level of neurite extension[30]. To investigate the effect of surface morphology on the attachment and differentiation of neuronal cells, titanium with different surface topographical features was used,

varying from smooth to nanostructured surfaces. The nanostructured surfaces of titanium specifically designed to prevent bacterial colonisation through contact killing was found to enhance the attachment of PC 12 cells and stimulate their differentiation. In contrast, smooth surfaces that did not contain nanoscale topographical features were not able to support adhesion and differentiation of PC 12 cells[33].

The extent of PC 12 differentiation in response to different stimuli is typically assessed using quantitative or semi-quantitative morphological methods, which include determination of the cell size, number of cells displaying neurite processes, and the neurite length[1,30]. Expression of specific proteins during the process of differentiation can be used as a marker for neurochemical activity[1]. Neurotypic proteins that have been associated with PC 12 differentiation and axonal elongation include the growth associated protein 43 (GAP−43), synapsin and synaptophysin, which are presynaptic membrane associated proteins[1]. Differentiation of PC 12 cells in response to NGF is induced by NGF acting on the receptor tyrosine kinase (RTK), TrkA[36], which then initiates several signalling cascades[2,4]. These include mitogen-activated protein kinase (Raf/MEK/MAPK) and PLC_{λ}/PKC pathways[1].

The contradictory finding about the role of *ras* and *src* oncogene products in improving rather than the blocking of NGF-induced differentiation has led to the identification of signalling pathways that involve both Ras and Src as a part of the signalling cascade that responds to NGF[4]. It was shown that the responses of cells to both NGF and the epidermal growth factor (EGF) required the use of the same pathway that involves extracellular regulated kinases (ERK) and MAPK. The activation of MAP kinase was found to be necessary for PC 12 cell differentiation as shown by using activated mutants of MAP kinase, kinase 1[37]. In addition, the neurotransmitter pituitary adenylate cyclase-activating polypeptide (PACAP) was shown to stimulate neurite outgrowth through ERK activation in a process that is comparable to but distinct from NGF signalling[4]. The very different outcomes due to EGF and NGF stimulation could arise from the duration of signalling through ERKs[4]. Rapid and transient Ras- and Rap1-dependent ERK phosphorylation is induced in response to EGF stimulation whereas NGF stimulation of ERK is both rapid and continuous, with sustained activation that is dependent on signalling to ERK via Rap1[4].

The phospho protein expression of GAP−43, an endogenous substrate for PKC, is stimulated in the presence of NGF in PC 12 cells. The upregulation of GAP−43 mRNA and the GAP−43 protein is linked to the PC 12 cells differentiating into neuronal-like cells[1]. Localisation of synaptophysin and synapsin will occur in the presynaptic membrane regulating the synaptic vesicle fusion and release of neurotransmitters[1]. Thus, actively differentiating PC 12 cells will exhibit an increase in the level of these proteins in the culture. When studying the expression of GAP−43 and synapsin I in differentiating PC 12 cells[1], it was found that the expression of GAP− 43 was low on day 0 and increased to maximum levels on day 5, whereas the expression of synapsin I increased relatively slowly on days 0−4 and then more rapidly on days 5−7[1].

PC 12 cell response to NGF

The use of PC 12 cells to study cellular responses to nerve growth factors (NGF) relies on the assumption that all PC 12 cells within a given population will respond to a NGF in a similar manner[7]. However, this assumption was challenged when it was found that in the first days of NGF exposure, the majority of the population is multiplying, with only a minority of the cells extending neurites[2,38]. These findings strongly suggested that PC 12 cells do not respond in an identical manner. On the contrary, the response appears to be differential and depend on the status of the cell and the parameter being investigated[7]. For example, it was observed that the addition of NGF alone to a serum free PC 12 cell culture does not cause a detectable increase in the cell number but stimulates neurite outgrowth at a rate that is significantly higher than that of cells stimulated by NGF while growing exponentially in full serum medium[38]. In an attempt to develop a more stable cell system that could be used to elucidate the mechanism of NGF action on cells, a set of experiments were designed to test whether serum starvation could be used for cell synchronization[7]. These experiments were also aimed to illustrate the cellular response of PC 12 to serum and NGF using selected markers for proliferation (*e.g.*, DNA synthesis and cell number) and differentiation (*e.g.*, neurite extension, appearance of the binding sites of saxitoxin, and downregulation of the epidermal growth factor receptor). Long term differentiation of PC 12 cells induced by the addition of NGF and observed for more than 14 days has displayed an increase in electrical excitability of cells. This was attributed to an increase in the amount of voltage-sensitive Na^+ channels as measured by saxitoxin binding[7]. The findings also revealed that serum starvation led to the accumulation of cells in the G_1 phase of the cell cycle. The effect of NGF on the cells accumulated in the G_1 phase was found to be more robust when compared to that in PC 12 cells that were exponentially growing when NGF was added. In the latter, only a certain number of cells were in the G_1 phase[7]. In light of these findings, it can be stated that PC 12 cells respond to NGF in a cell cycle-specific manner[7]. Furthermore, it has been experimentally demonstrated that the extent of neurite extension is NGF concentration-dependent. When different concentrations of NGF (0, 25, 50 and 100 ng/mL) were added to low serum medium, the extent of neurite extension wasthe strongest in the presence

of a NGF concentration of 50 ng/mL[30], confirming that PC 12 cells respond differently to different concentrations of NGF. Furthermore, this study provided additional confirmation that the differentiation of PC 12 cells into neuron-like structures was accompanied by an arrest of the PC 12 cells in the post-mitotic G_0 stage of the cell cycle, shown using MTS assay. The effect of THz radiation exposure on PC 12 cell differentiation was studied by treating PC 12 cells with NGF and monitoring the neurite outgrowth for up to 7 days[16]. The THz treated PC 12 cells underwent neuronal differentiation, with 86.17 ± 4.06% of the population extending neurites from 0 – 20 μm in length, while 14.90 ± 4.88% of the cell population extended neurites of 20 – 40 μm in length[16]. The untreated control sample consisted of a population of 65.91 ± 5.04% with neurite lengths in the range of 0 – 20 μm, while 23.86 ± 3.48% had neurites in the length of 20 – 40 μm. In comparison to the control, the THz-treated cells demonstrated more neurites in the 0 – 20 μm range on day 7 and less in the range of 20 – 40 μm[16].

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